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(54) Title: CHEMOKINES AS ADJUVANTS OF IMMUNE RESPONSE

(57) Abstract

Dendritic cells play a critical role in antigen-specific immune responses. Materials and methods are provided for treating disease states, including cancer and autoimmune disease, by facilitating or inhibiting the migration or activation of antigen-presenting dendritic cells. In particular, chemokines are used to initiate, amplify or modulate an immune response. In one emodiment, chemokines are used to attract dentritic cells to the site of antigen delivery. An increase number of dendritic at the site of antigen delivery means more antigen uptake and a modified immune response.

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CHEMOKINES AS ADJUVANTS OF IMMUNE RESPONSE

Field Of The Invention

The invention relates to the use of human chemokines in the treatment of disease states, including cancer. The administered chemokines direct the migration of either all antigen-presenting dendritic cells or a specific subset of dentritic cells. In one embodiment, disease-specific antigen(s) and/or a moiety designed to activate dentritic cells is administered in conjunction with the chemokine(s).

Background Of The Invention

Dendritic cells (DC) specialize in the uptake of antigen and their presentation to T cells. DC thus play a critical role in antigen-specific immune responses.

DC are represented by a diverse population of morphologically similar cell types distributed widely throughout the body in a variety of lymphoid and non-lymphoid tissues (Caux, *et al.*, 1995, *Immunology Today* **16**:2; Steinman, 1991, *Ann. Rev. Immunol.* **9**:271-296). These cells include lymphoid DC of the spleen, Langerhans cells of the epidermis, and veiled cells in the blood circulation. DC are collectively classified as a group based on their morphology, high levels of surface MHC-class II expression as well as several accessory molecules (B7-1[CD80] and B7-2[CD86]) that mediate T cell binding and costimulation (Inaba, *et al.*, 1990, *Intern. Rev. Immunol.* **6**:197-206; Frendenthal, *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* **87**:7698), and absence of certain other surface markers expressed on T cells, B cells, monocytes, and natural killer cells.

DC are bone marrow-derived and migrate as precursors through blood stream to tissues, where they become resident cells such as Langerhans cells in the epidermis.

In the periphery, following pathogen invasion, immature DC such as fresh Langerhans cells are recruited at the site of inflammation (Kaplan, *et al.*, 1992, *J. Exp. Med.* **175**:1717-1728; McWilliam, *et al.*, 1994, *J. Exp. Med.*

179:1331-1336) where they capture and process antigens, (Inaba, *et al.*, 1986. *J. Exp. Med.* **164**:605-613; Streilein, *et al.*, 1989, *J. Immunol.* **143**:3925-3933; Romani, *et al.*, 1989., *J. Exp. Med.* **169**:1169-1178; Puré, *et al.*, 1990. *J. Exp. Med.* **172**:1459-1469; Schuler, *et al.*, 1985, *J. Exp. Med.* **161**:526-546).

Antigen-loaded DC then migrate from the peripheral tissue via the lymphatics to the T cell rich area of lymph nodes, where the mature DC are called interdigitating cells (IDC) (Austyn, *et al.*, 1988, *J. Exp. Med.* **167**:646-651; Kupiec-Weglinski, *et al.*, 1988, *J. Exp. Med.* **167**:632-645; Larsen, *et al.*, 1990, *J. Exp. Med.* **172**:1483-1494; Fossum, S. 1988, *Scand. J. Immunol.* **27**:97-105; Macatonia, *et al.*, 1987, *J. Exp. Med.* **166**:1654-1667; Kripke, *et al.*, 1990., *J. Immunol.* **145**:2833-2838). At this site, they present the processed antigens to naive T cells and generate an antigen-specific primary T cell response (Liu, *et al.*, 1993, *J. Exp. Med.* **177**:1299-1307; Sornasse, *et al.*, 1992, *J. Exp. Med.* **175**:15-21; Heufler, *et al.*, 1988. , *J. Exp. Med.* **167**:700-705).

During their migration from peripheral tissues to lymphoid organs, DC undergo a maturation process encompassing dramatic changes in phenotype and functions (Larsen, *et al.*, 1990, *J. Exp. Med.* **172**:1483-1494; Streilein, *et al.*, 1990, *Immunol. Rev.* **117**:159-184; De Smedt, *et al.*, 1996, *J. Exp. Med.* **184**:1413-1424). In particular, in contrast to immature DC such as fresh Langerhans cells, which capture and process soluble proteins efficiently and are effective at activating specific memory and effector T cells, mature DC such as IDC of lymphoid organs are poor in antigen capture and processing but markedly efficient in naive T cell priming (Inaba, *et al.*, 1986. *J. Exp. Med.* **164**:605-613; Streilein, *et al.*, 1989, *J. Immunol.* **143**:3925-3933; Romani, *et al.*, 1989, *J. Exp. Med.* **169**:1169-1178; Puré, *et al.*, 1990, *J. Exp. Med.* **172**:1459-1469; Sallusto, *et al.*, 1995, *J. Exp. Med.* **182**:389-400; Cella, *et al.*, 1997, *Current Opin. Immunol.* **9**:10-16).

Signals regulating the traffic pattern of DC are complex and not fully understood.

Signals provided by TNFα and LPS are known to induce *in vivo* migration of resident DC from the tissues to the draining lymphoid organs (De Smedt, *et al.*, 1996, *J. Exp. Med.* **184**:1413-1424; MacPherson, *et al.*, 1995, *J. Immunol.* **154**:1317-1322; Roake, *et al.*, 1995, *J. Exp. Med.* **181**:2237-2247; Cumberbatch *et al.*, 1992, *Immunology.* **75**:257-263; Cumberbatch, *et al.*, 1995, *Immunology.* **84**:31-35).

Chemokines are small molecular weight proteins that regulate leukocyte migration and activation (Oppenheim, 1993, *Adv. Exp. Med. Biol.* **351**:183-186; Schall, *et al.*, 1994, *Curr. Opin. Immunol.* **6**:865-873; Rollins, 1997, *Blood* **90**:909-928; Baggiolini, *et al.*, 1994, *Adv. Immunol.* **55**:97-179). They are secreted by activated leukocytes themselves, and by stromal cells including endothelial cells and epithelial cells upon inflammatory stimuli (Oppenheim, 1993, *Adv. Exp. Med. Biol.* **351**:183-186; Schall, *et al.*, 1994, *Curr. Opin. Immunol.* **6**:865-873; Rollins, 1997, *Blood* **90**:909-928; Baggiolini, *et al.*, 1994, *Adv. Immunol.* **55**:97-179). Responses to chemokines are mediated by seven transmembrane spanning G-protein-coupled receptors (Rollins, 1997, *Blood* **90**:909-928; Premack, *et al.*, 1996, *Nat. Med.* **2**:1174-1178; Murphy, P.M. 1994, *Ann. Rev. Immunol.* **12**:593-633). Several chemokines such as monocyte chemotactic protein (MCP)-3, MCP-4, macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES (regualted on activation, normal T cell expressed and secreted), SDF-1, Teck (thymus expressed chemokine) and MDC (marcrophage derived chemokine) have been reported to attract DC *in vitro* (Sozzani, *et al.*, 1995, *J. Immunol.* **155**:3292-3295; Sozzani, *et al.*, 1997, *J. Immunol.* **159**:1993-2000; Xu, *et al.*, 1996, *J. Leukoc. Biol.* **60**:365-371; MacPherson, *et al.*, 1995, *J. Immunol.* **154**:1317-1322; Roake, *et al.*, 1995, *J. Exp. Med.* **181**:2237-2247).

In recent years, investigators have attempted to exploit the activity of DC in the treatment of cancer. In an animal model, as few as 2 x 10^5 antigen-pulsed DC will induce immunity when injected into naive mice (Inaba *at al.*, 1990, *Intern. Rev. Immunol.* **6**:197-206). Flamand *et al.* (*Eur. J. Immunol.*, 1994, **24**:605-610) pulsed mouse DC with the idiotype antigen from a B-cell lymphoma and injected them into naive mice. This treatment effectively protected the recipient mice from subsequent tumor challenges and established a state of lasting immunity. Injection of antigen alone, or B cells pulsed with antigen, had no effect, suggesting that it was the unique characteristics of DC that were responsible for the anti-tumor response. It has been postulated that DC are not only capable of inducing anti-tumor immunity, but that they are absolutely essential for this process to occur (Ostrand-Rosenberg, 1994, *Current Opinion in Immunol.* **6**:722-727; Grabbe *et al.*, 1995, *Immunol. Today* **16**:117-120; Huang *et al.*, 1994, *Science* **264**:961-965). Huang and coworkers (Huang *et al.*, 1994, *Science* **264**:961-965) inoculated mice with a B7-1 transfected tumor that was known to produce anti-tumor immunity. They demonstrated that only mice with MHC-compatible APC

were capable of rejecting a tumor challenge. Studies in humans have demonstrated a similar role for DC. It has been reported that peptide-specific CTL are readily induced from purified CD8+ T cells using peptide-pulsed DC, but are not elicited when peptide-pulsed monocytes are used (Mehta-Damani *et al.*, 1994, *J. Immunology* **153**:996-1003).

Of significant clinical interest, the histologic infiltration of dendritic cells into primary tumor lesions has been associated with significantly prolonged patient survival and a reduced incidence of metastatic disease in patients with bladder, lung, esophageal, gastric and nasopharygeal carcinoma. In contrast, a comparatively poorer clinical prognosis is observed for patients with lesions that exhibit a sparse infiltration with DC and metastatic lesions are frequently deficient in DC infiltration (Becker, 1993, *In Vivo* 7:187; Zeid *et al.*, 1993, *Pathology* **25**:338; Furihaton *et al.*, 1992, **61**:409; Tsujitani *et al.*, 1990, *Cancer* **66**:2012; Gianni *et al.*, 1991, *Pathol. Res. Pract.* **187**:496; Murphy *et al.*, 1993, *J. Inv. Dermatol.* **100**:3358). A patient with advanced B-cell lymphoma was recently treated with DC pulsed with the patient's own tumor idiotype (Hsu *et al.*, 1996, *Nature Medicine* **2**(1):52). This produced a measurable reduction in the patient's B-cell lymphoma. Treatment of prostate cancer using DC pulsed with PSM antigen has been reported by Murphy *et al.* (*The Prostate* 1996 **29**:371).

Techniques have recently emerged for the *in vitro* propagation of large numbers of DC from circulating monocytes or from CD34 hematopoietic progenitors in response to granulocyte-macrophage colony stimulating factor (GM-CSF) in combination with either interleukin 4 (IL-4) or tissue necrosis factor α (TNFα) (Sallusto *et al.*, 1994, *J. Exp. Med.* **179**:1109-1118; Romani *et al.*, 1994, *J. Exp. Med.* **180**:83-93: Caux *et al.*, 1992, *Nature* **360**:258). The combination of GM-CSF and IL-4 induces peripheral blood monocytes to differentiate into potent DC (Kiertscher and Roth, 1996, *J. Leukocyte Biol.* **59**:208-281). With the combination of these two cytokines a 100-fold increase in the yield of DC can be achieved from peripheral blood *in vitro*.

In mice, tumor antigen-loaded *in vitro* generated DC have been shown, by various groups, to prevent the development of tumors and more importantly to induce the regression of established tumors. A clinical trial has been conducted in which patients with melanoma are being treated with GM-CSF-activated APC pulsed with a peptide from the MAGE-1 tumor

antigen (Mehta-Damani, *et al.*, 1994, *J. Immunology* **153**:996-1003). Pre-immunization, tumor-infiltrating lymphocytes from two patients were predominantly $CD4^+$ and lacked specific tumor reactivity. In contrast, after immunization tumor infiltrating lymphocytes from the same patients were predominantly $CD8^+$ and demonstrated MAGE-1 specific anti-tumor cytotoxicity. It thus appears from these studies that DC have a unique and potent capacity to stimulate immune responses.

Dendritic cell therapy thus represents a very promising approach to the treatment of disease, in particular, cancer. There is a continuing need for improved materials and methods that can be used not only to expand and activate antigen presenting dendritic cells, but to facilitate the migration of DC so as to be both therapeutically as well as prophylactically useful.

Summary of the Invention

The present invention fulfills the foregoing need by providing materials and methods for treating disease states by facilitating or inhibiting the migration or activation of antigen-presenting dendritic cells. It has now been discovered that chemokines are useful therapeutic agents. Disease states which can be treated in accordance with the invention include parasitic infections, bacterial infections, viral infections, fungal infections, cancer, autoimmune dieseases, graft rejection and allergy.

The invention provides a method of treating disease states comprising administering to an individual in need thereof an amount of chemokine sufficient to increase the migration of immature dendritic cells to the site of antigen delivery. In one aspect of the invention a chemokine such as MIP-3α, MIP-1α and RANTES, or a combination thereof is administered. In a preferred method of the invention, a disease-associated antigen, such as a tumor-associated antigen is administered in conjunction with the chemokine.

Another aspect of the invention provides a method of treating disease states comprising administering to an individual in need thereof an amount of chemokine sufficient to decrease the migration of immature dendritic cells to the site of antigen delivery.

In still another aspect of the invention, cytokines, in particular GM-CSF and IL-4 are administrered in combination, either before or concurently,

with the chemokine. Administration of GM-CSF and IL-4 stimulates generation of DC from precursors, thereby increasing the number of DC available to capture and process antigen.

Yet another aspect of the invention an activating agent such as TNF-α is administered to provide maturation signals which drives the migration of DC from tissues toward lypmph organs vessels to the draining lymph.

Brief Description of the Drawings

Figure 1 shows that immunization with a plasmid encoding a chemokine and a tumor-associated antigen has a protective effect against tumor engraftment.

Figure 2 shows that greater CTL activeity with the administration of a chemokine

Detailed Description of the Invention

All references cited herein are incorporated in their entirety by reference.

The relation between signals inducing DC migration *in vivo* and their responses to chemokines was heretofore not known. The inventors have discovered that the pattern of chemokine receptors expressed by DC change according to their stage of maturation and that chemokines can be used to drive migration of DC subsets and thereby control the initiation of the immune response. Chemokines can be used in accordance with the invention as adjuvants to attract selectively the immature DC subsets at the site of antigen delivery. In the context of autoimmune disease, tissue rejection or allergy, the invention provides a method of blocking DC functions by interfering with their migration through e.g., the development of CCR6 and CCR7 antagonists.

Depending on the subset of DC presenting the antigen to the immune system, the response could vary dramatically. DC can induce tolerance. DC found in the medulla of the thymus play a role in the negative selection of developing self-reactive thymocytes (Brocker, *et al.*, 1997, *J. Exp. Med.*

185(3):541-550). DC can also tolerize self-reactive peripheral T cells (Kurts, *et al.*, 1997, *J. Exp. Med.* **186**(2):239-245; Adler, *et al.*, 1998, *J. Exp. Med.* **187**(10):1555-1564). A specific subset of mouse DC, possibly of lymphoid origin, has been proposed to induce immune tolerance (Ardavin, 1993, *Nature* **362**(6422):761-763). Furthermore, the recent description that the candidate human counterpart to the lymphoid DC (the DC-2) (Grouard, *et al.*, 1997, *J. Exp. Med.* **185**(6):1101-1111) cannot secrete IL-12 suggests that, following presentation by this subpopulation, the immune response might be biased towards a TH-2 type.

When the goal is to decrease the immune response, tolerizing DC (autoimmunity, allergy) are recruited, or the quality of the response is modified by recruiting specifically DC-2 (TH1 greater that TH2, i.e., in allergy).

A chemokine for use in the invention is a natural protein of the body that is active on a restricted subset of DC, in particular, immature DC. Several of these cytokines, including MIP-3α, Teck, DCtactin-β and MCP-4, have been identified by the inventors.

The chemokine used in practicing the invention may be a recombinant protein with an amino-acid sequence identical to the natural product, or a recombinant protein derived from the natural product but including modifications that changes its pharmacokinetic properties while keeping its original chemoattractant property. The mode of delivery of the chemokine may be by injection, including intradermal, intramuscular and subcutaneous, or topical, such as an ointment or a patch.

The chemokine may also be delivered as a nucleic acid sequence by the way of a vector, such as a viral vector (e.g., adenovirus, poxvirus, retrovirus, lentivirus), or an engineered plasmid DNA.

The term "chemokine" as used herein includes chemotactic agents. A chemotactic agent may be a small chemical compound which is a selective

agonist of a chemokine receptor expressed by immature DC. CCR6, the natural receptor the the chemokine MIP-3α is an example of such a receptor.

In a particularly preferred embodiment of the invention, the chemokine is administered with a disease-associated antigen. The antigen can be any molecular moiety against which an increase or decrese in immune response is sought. This includes antigens derived from organisms known to cause diseases in man or animal such as bacteria, viruses, parasites (e.g., *Leishmania*) and fungi. This also includes antigens expressed by tumors (tumor-associated antigens) and plant antigens (allergens).

Tumor associated antigens for use in the invention include, but are not limited to Melan-A, tyrosinase, p97, β-HCG, GalNAc, MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-12, MART-1, MUC1, MUC2, MUC3, MUC4, MUC18, CEA, DDC, melanoma antigen gp75, HKer 8, high molecular weight melanoma antigen, K19, Tyrl and Tyr2, members of the pMel 17 gene family, c-Met, PSA, PSM, α-fetoprotein, thyroperoxidase, gp100 and p53. This list is not intended to be exhaustive, but merely exemplary of the types of antigen which may be used in the practice of the invention.

Different combinations of antigens may be used that show optimal function with different ethnic groups, sex, geographic distributions, and stage of disease. In one embodiment of the invention at least two or more different antigens are administered in conjunction with the administration of chemokine.

The antigen can by delivered or administered at the same site and the same time as the chemokine, or after a delay not exceeding 48 hours. Concurrent or combined administration, as used herein means the chemokine and antigen are administered to the subject either (a) simultaneously in time, or (b) at different times during the course of a common treatment schedule. In the latter case, the two compounds are administered sufficiently close in time to achieve the intended effect. The antigen can be in the form of a protein, or one or several peptides, or of a nucleic acid sequence included in a delivery vector.

Both primary and metastatic cancer can be treated in accordance with the invention. Types of cancers which can be treated include but are not

limited to melanoma, breast, pancreatic, colon, lung, glioma, hepatocellular, endometrial, gastric, intestinal, renal, prostate, thyroid, ovarian, testicular, liver, head and neck, colorectal, esophagus, stomach, eye, bladder, glioblastoma, and metastatic carcinomas. The term "carcinoma" refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. Metastatic, as this term is used herein, is defined as the spread of tumor to a site distant to regional lymph nodes.

A moiety designed to activate, induce or stimulate maturity of the DC may be advantageously administered. Such agents provide maturation signals which promote migration from the tissues to the lymph nodes. This moiety can be a natural product of the body such as TNF-α or RP-105, or an agonist antibody recongnizing a specific structure on DC such as an anti-CD-40 antibody, or another substance. The activating substance can be a sequence of nucleic acids containing unmethylated CpG motifs known to stimulate DC. In the embodiment of the invention where the chemokine and/or antigen is delivered by the means of a plasmid vector, these nucleic acid sequences may be part of the vector.

GM-CSF and IL-4 can advantageous be administered in combination with the chemokine and/or antigen. The administration combination of GM-CSF and IL-4 stimulates generation of DC from precursors. GM-CSF and IL-4 may be administered for purposes of increasing the number of circulating immature DC which might then be locally recruited locally be the subsequent injection of chemokine(s). This protocol would imply a systemic pre-treatment for a least five to seven days with GM-CSF and IL-4. An alternative would be to favor by local administration of GM-CSF and IL-4 the local differentiation of DC-precursors (monocytes) into immature DC which could then pick up the antigen delivered at the same site.

Generally, chemokine(s) and/or antigen(s) and/or activating agent(s) and/or cytokine(s) are administered as pharmaceutical compositions comprising an effective amount of chemokine(s) and/or antigen(s) and/or

activating agent(s) and/or cytokine(s) in a pharmaceutical carrier. These reagents can be combined for therapeutic use with additional active or inert ingredients, e.g., in conventional pharmaceutically acceptable carriers or diluents, e.g., immunogenic adjuvants, along with physiologically innocuous stabilizers and excipients. A pharmaceutical carrier can be any compatible, non-toxic substance suitable for delivering the compositions of the invention to a patient.

The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medicants administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Animal testing of effective doses for treatment of particular cancers will provide further predictive indication of human dosage. Various considerations are described, e.g., in Gilman *et al.* (eds.) (1990) *Goodman and Gilman's: The Pharmacological Bases of Therapeutics*, 8th Ed., Pergamon Press; and *Remington's Pharmaceutical Sciences*, 17th ed. (1990), Mack Publishing Co., Easton, PA. Methods for administration are discussed therein and below, e.g., for intravenous, intraperitoneal, or intramuscular administration, transdermal diffusion, and others. Pharmaceutically acceptable carriers will include water, saline, buffers, and other compounds described, e.g., in the *Merck Index*, Merck & Co., Rahway, New Jersey. Slow release formulations, or a slow release apparatus may be used for continuous administration.

Dosage ranges for chemokine(s) and/or antigen(s) and/or activating agent(s) would ordinarily be expected to be in amounts lower than 1 mM concentrations, typically less than about 10 μM concentrations, usually less than about 100 nM, preferably less than about 10 pM (picomolar), and most preferably less than about 1 fM (femtomolar), with an appropriate carrier. Generally, treatment is initiated with smaller doages which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstance is reached. Determination of the proper dosage and administration regime for a particular situation is within the skill of the art.

The preferred biologically active dose of GM-CSF and IL-4 in the practice of the claimed invention is that dosing combination which will

induce maximum increase in the number of circulating $CD14^+/CD13^+$ precursor cells; the expression of antigen presenting molecules on the surface of DC precursors and mature DC; antigen presenting activity to T cells; and/or stimulation of antigen-dependent T cell response consistent with mature DC function. In the practice of the invention the amount of IL-4 to be used for subcutaneously administration typically ranges from about 0.05 to about 8.0μg/kg/day, preferably 0.25 - 6.0 μg/kg/day, most preferably 0.50 - 4.0 μg/kg/day. The amount of GM-CSF is to be used for subcutaneous administration typically ranges from about 0.25μg/kg/day to about 10.0 μg/kg/day, preferably from about 1.0 - 8.0 μg/kg/day, most preferably 2.5 - 5.0μg/kg/day. An effective amount for a particular patient can be established by measuring a significant change in one or more of the parameters indicated above.

EXAMPLES

The invention can be illustrated by way of the following non-limiting examples, which can be more easily understood by reference to the following materials and methods.

Hematopoietic factors, reagents and cell lines. Recombinant GM-CSF (specific activity: 2.10^6 U/mg, Schering-Plough Research Institute, Kenilworth, NJ) was used at a saturating concentration of 100 ng/ml. Recombinant human TNFα (specific activity: $2x10^7$ U/mg, Genzyme, Boston, MA) was used at an optimal concentration of 2.5 ng/m. Recombinant human SCF (specific activity: $4x10^5$ U/mg, R&D Abington, UK) was used at an optimal concentration of 25 ng/ml. Recombinant human IL-4 (specific activity: 2.10^7 U/mg, Schering-Plough Research Institute, Kenilworth, NJ) was used at a saturating concentration of 50 U/ml. Recombinant human chemokines MIP-1α (specific activity: $2x10^5$ U/mg, $9x10^{12}$ U/M), RANTES (specific activity: $1x10^4$ U/mg, $8x10^{10}$ U/M), MIP-3α (specific activity: $4x10^5$ U/mg, $3x10^{12}$ U/M) and MIP-3β (specific activity: $1x10^4$ U/mg, $9x10^{10}$ U/M) were obtained through R&D (Abington, UK). LPS was used at 10 ng/ml (Sigma).

The murine CD40 ligand transfected cell line (CD40-L L cells) was used as stimulator of DC maturation.

Generation of DC from cord blood CD34+ HPC. Umbilical cord blood samples were obtained following full term delivery. Cells bearing CD34+ antigen were isolated from mononuclear fractions through positive selection as described (Caux, *et al.*, 1996, *J. Exp. Med.* **184**:695-706; Caux, *et al.*, 1990, *Blood.* **75**:2292-2298), using anti-CD34+ monoclonal antibody (Immu-133.3, Immunotech Marseille, France), goat anti mouse IgG coated microbeads (Miltenyi Biotec GmBH, Bergish Gladbach, Germany) and Minimacs separation columns (Miltenyi Biotec). In all experiments the isolated cells were 80% to 99% CD34+. After purification, CD34+ cells were cryopreserved in 10% DMSO.

Cultures were established in the presence of SCF, GM-CSF and TNFα as described (Caux, *et al.*, 1996, *J. Exp. Med.* **184**:695-706) in endotoxin-free medium consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Life Techniques, France, Irvine, UK), 10 mM Hepes, 2 mM L-glutamine, $5x10^{-5}$ M β-mercaptoethanol, 100 μg/ml gentamicin (Schering-Plough, Levallois, France) (referred to as complete medium). After thawing, CD34+ cells were seeded for expansion in 25 to 75 cm^2 culture vessels (Linbro, ICN Biomedicals, Acron, OH) at $2x10^4$ cells/ml. Optimal conditions were maintained by splitting these cultures at day 5 and 10 with medium containing fresh GM-CSF and TNFα (cell concentration: $1\text{-}3x10^5$ cells/ml). At day 12, between 70 to 90% of the cells are CD1a+ DC.

Isolation of immature and mature DC according to CD86 expression by FACS-sorting. After 12 days of culture in presence of GM-CSF and TNFα, cells were collected and labelled with FITC-conjugated OKT6 (CD1a) (Ortho Diagnosis System, Raritan, NJ) and PE-conjugated IT2.2 (CD86) (Pharmingen, San Diego, CA). Cells were separated according to CD1a and CD86 expression into immature CD1a+CD86-, and mature CD1a+CD86+ DC populations using a FACStarplus® (laser setting: power 250 mW, excitation wavelength 488 nm, Becton-Dickinson, Sunnyvale, CA). All the procedures of staining and sorting were performed in presence of 0.5 mM EDTA in order to avoid cell aggregation. Reanalysis of the sorted populations showed a purity > 98%.

Generation of DC from peripheral blood monocytes. Monocytes were purified by immunomagnetic depletion (Dynabeads, Dynal Oslo, Norway)

after preparation of PBMC followed by a 52% Percoll gradient. The depletion was performed with anti-CD3 (OKT3), anti-CD19 (4G2), anti-CD8 (OKT8), anti-CD56 (NKH1, Coulter Corporation, Hialeah, FL) and anti-CD16 (ION16, Immunotech) monoclonal antibodies. Monocyte-derived dendritic cells were produced by culturing purified monocytes for 6-7 days in the presence of GM-CSF and IL-4 (Sallusto, *et al.*, 1994, *J. Exp. Med.* **179**:1109-1118).

Induction of maturation of in vitro generated DC. $CD34^+$ HPC were cultured until day 6 in presence of GM-CSF+TNFα and in presence of GM-CSF alone from day 6 to day 12 in order to preserve their immaturity. Immature DC from $CD34^+$ HPC or monocyte-derived DC were activated for 3h to 72h in presence of TNFα (2.5 ng/ml) or LPS (10 ng/ml) or CD40L transfected L cells (1 L cells for 5 DC) as described (Caux, *et al.*, 1994, *J. Exp. Med.* **180**:1263-1272) .

Purification of $CD11c^+$ DC from peripheral blood or tonsils. $CD11c^+$ DC were prepared as previously described from peripheral blood or tonsils (Grouard, *et al.*, 1996, *Nature* **384**:364-367). Briefly, tonsils obtained from children undergoing tonsillectomy were finely minced and digested with collagenase IV and DNase I (Sigma). The collected cells were centrifuged through Ficoll-Hypaque with SRBC (BioMérieux, Lyon, France) for 15 min at 500 rpm, then for 30 min at 2000 rpm. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque. $CD3^+$ T cells (OKT3), $CD19^+$ B cells (4G7), and $CD14^+$ monocytes (MOP9) were removed from the resulting low density cells by magnetic beads (anti-mouse Ig-coated Dynabeads, Dynal). A second depletion was performed with anti-NKH1, anti-glycophorine A (Immunotech) and anti-CD20 (1F54). The remaining cells were stained with the following mAbs: anti-CD1a FITC (OKT6); anti-CD14 FITC, anti-CD57 FITC, anti-CD16 FITC, anti-CD7 FITC, anti-CD20 FITC, anti-CD3 FITC (Becton Dickinson, Mountain View, CA); anti-CD4 PE-Cy5 (Immunotech) and anti-CD11c PE (Becton Dickinson). $CD4^+CD11c^+lineage^-$ DC were isolated by cell sorting using a FACStarPlus® (laser setting: power 250 mW, excitation wavelength 488 nm). All the procedures of depletion, staining and sorting were performed in presence of 0.5 mM EDTA. Reanalysis of the sorted population showed a purity > 97%.

Chemotaxis assay. Cell migration was evaluated using a chemotaxis microchamber technique (48-well Boyden microchamber, Neuroprobe, Pleasanton, CA) (Bacon, *et al.*, 1988, *Br. J. Pharmacol.* **95**:966-974). Briefly,

human recombinant MIP-3α and MIP-3β, MIP-1α and RANTES were diluted to concentrations ranging from 1 ng/ml to 1000 ng/ml in RPMI 1640 medium, and were added to the lower wells of the chemotaxis chamber. 10^5 cells/well (or $5x10^4$ cells/well for $CD11c^+$ DC) in 50μl of RPMI 1640 medium were applied to the upper wells of the chamber, with a standard 5-μm pore polyvinylpyrrolidone-free polycarbonate filter (Neuroprobe) separating the lower wells. The chamber was incubated at 37°C in humidified air with 5% CO_2 for 1h. Then, cells which had migrated to the underside of the filter were stained with Field's A and Field's B (BDH, Dorcet, England) and counted using an image analyser (software: Vision Explorer and ETC 3000, Graphtek, Mirmande, France) in two randomly selected low power fields (magification x20). Each assay was performed in duplicate and the results were expressed as the mean ± SD of migrating cells per 2 fields.

Extraction of total RNA and Synthesis of cDNA. Cells were prepared as described above, and total RNA was extracted by the guanidinium thiocyanate method as mentioned by the manufacturer (RNAgents total RNA isolation system, Promega). After DNAse I (RQ1 RNAse free DNAse, Promega) treatement, RNA was quantified by spectrophotometry and the quality was evaluated by electrophoresis in formaldehyde denaturing conditions. First strand cDNA was synthetized from total RNA extracted in RNAse-free conditions. The reaction was performed with 5 μg of total RNA, 25 ng/μl oligo dT_{12-18} primers (Pharmacia, Orsay, France) and the Superscript kit (SuperScript II RNase H^- Reverse Transcripase, Gibco BRL), as described by the manufacturer. For all samples, synthesis of cDNA was controlled and calibrated by RT-PCR using β-actin primers for 21 cycles.

RT-PCR analysis. Semi-quantitative PCR was performed in a Perkin Elmer 9600 thermal cycler, in a final volume of 100 μl reaction mixture containing 2.5 U AmpliTaq enzyme (5U/μl, Perkin Elmer, Paris, France) with its 1X buffer, 0.2 mM of each dNTP (Perkin Elmer, Paris, France), 5% DMSO, and 1 μM of each forward and reverse primers. CCR6 (Accession No. Z79784) and CCR7 (Accession No. L08176) primers were designed within regions of lowest homology between the chemokine receptors. +80/CCR6 5'- ATTTCAGCGATGTTTTCGACTC -3' forward primer, -1081/CCR6 5'- GGAGAAGCCTGAGGACTTGTA -3' reverse primer, +154/CCR7 5'- GATTACATCGGAGACAACACC -3' forward primer and -1202/CCR7 5'- TAGTCCAGGCAGAAGAGTCG -3' reverse primer were used for RT-PCR and sequencing. For both chemokine receptors, the reaction mixture was

subjected to 30 and 35 cycles of PCR with the following conditions : 94°C for 1 min, 61.5°C for 2 min and 72°C for 3 min. PCR products were visualized on 1.2% agarose gels containing 0.5 μg/ml ethidium bromide. Reaction products migrating at the predicted size (1,021 bp for CCR6 and 1,067 bp for CCR7) were gel purified and subcloned into pCRII TA cloning vector (Invitrogen, Leek, The Netherlands) for sequencing verification on an ABI 373A Sequencer (Applied Biosystems, Foster City, CA.) using dye terminator technology. Two other oligonucleotides, -622/CCR6 5'- GCTGCCTTGGGTGTTGTATTT -3' and +662/CCR7 5'- AGAGGAGCAGCAGTGAGCAA -3', were used as probes for hybridization with the PCR products separated on 1.2% agarose gel and blotted onto Hybond N^+ membranes (Amersham, Les Ulis, France).

Calcium fluorimetry. Intracellular Ca^{2+} concentration was measured using the fluorescent probe Indo-1, according to the technique reported by Grynkiewicz *et al.* (*J. Biol. Chem.*, 1985, **260**:3440-3450) Briefly, cells were washed in PBS and resuspended at 10^7 cells/ml in complete RPMI 1640 medium (see above). Then, cells were incubated for 45 min at room temperature with 3 μg/ml Indo-1 AM (Molecular Probes) in the dark. After incubation, cells were washed and resuspended in HBSS/1% FCS at 10^7 cells/ml. Before measurement of intracellular Ca^{2+} concentration, cells were diluted 10 fold in HBSS/10 mM Hepes/1.6 mM $CaCl_2$ preheated at 39°C. Samples were excited at 330 nm with continous stiring and the Indo-1 fluorescence was measured as a function of time at 405 nm (dye is complexed with Ca^{2+}) and 485 nm (Ca^{2+}-free medium), in a 810 Photomultiplier Detection System (software: Felix, Photon Technology International, Monmouth Junction, NJ). Results are expressed as the ratio of values obtained at the two emission wavelengths.

In situ hybridization. In situ hybridization was performed as described (Peuchmaur, *et al.*, 1990, *Am. J. Pathol.* **136**:383-390). Two couple primers were used for amplifying by RT-PCR the majority of the open reading frame of MIP-3α (Accession No. D86955) and MIP-3β 3α (Accession No. U77180) genes. +77/MIP-3α 5'- TTGCTCCTGGCTGCTTTG -3' forward primer and -425/MIP-3α 5'- ACCCTCCATGATGTGCAAG -3' reverse primer, +25/MIP-3β 5'- CTGCTGGTTCTCTGGACTTC -3' forward primer and -439/MIP-3β 5'- CACACTCACACTCACACACAC -3' reverse primer, were used as described above with an annealing temperature at 62°C. Then, PCR products were cloned into pCRII TA cloning vector (Invitrogen, Leek, The

Netherlands) for the generation of sense and anti-sense probes with the adapted promoters. Sense and antisense ^{35}S-labeled probes of MIP-3α and MIP-3β, were obtained by run off transcription of the 367 bp and 435 bp fragments, respectively. Six μm human tonsil sections were fixed in acetone and 4% paraformaldehyde followed by 0.1 M triethanolamine/0.25% acetic anhydride. The sections were hybridized overnight, RNAse A treated and exposed for 24 days. After development sections were stained with hematoxylin.

Example 1

Differential responsiveness to MIP-3α and MIP-3β during development of CD34⁺-derived DC

To understand the regulation of DC traffic the response to various chemokines of DC at different stages of maturation was studied. DC were generated from $CD34^+$ HPC cultured in the presence of GM-CSF+ TNFα, and tested at different days of culture for their ability to migrate in response to chemokines in Boyden microchambers. MIP-3α and MIP-3β recruited 2 to 3 times more $CD34^+$-derived DC than MIP-1α or RANTES. However, MIP-3α and MIP-3β attracted DC collected at different time points of the culture. The response to MIP-3α was already detected at day 4, maximal at day 5-6 and lasted until day 10. At day 13 to 14, the response to MIP-3α was usually lost. In contrast, the response to MIP-3β, which could not be detected before day 10, peaked at day 13, and persisted beyond day 15. Of note, at early time points, when most of the cells in culture were still DC precursors ($CD1a^-$ $CD86^-$), the response to MIP-3α could be detected at concentrations of 1 to 10 ng/ml (depending on the experiment). In contrast, four days later, when almost all cells were immature DC ($CD1a^+CD86^-$), ≥300 ng/ml were needed to attract the cells (Fig.2, day 10), suggesting a progressive desensitization of the cells during maturation. Relatively high concentrations of MIP-3β (300 ng/ml) were also needed to recruit mature DC ($CD1a^+CD86^+$). Checkerboard analysis established that MIP-3α and MIP-3β induced chemotaxis and not chemokinesis of DC.

To confirm the relation between the stage of maturation and the response to MIP-3α and MIP-3β, $CD34^+$-derived DC were sorted by FACS at day 10 of culture according to CD86 expression into immature DC ($CD1a^+CD86^-$) and mature DC ($CD1a^+CD86^+$). $CD1a^+CD86^-$ responded exclusively to MIP-3α while $CD1a^+CD86^+$ responded mainly to MIP-3β.

These observations also confirmed that the cells recruited by MIP-3α and MIP-3β were indeed DC ($CD1a^+$). The correlation between DC maturation and chemokine responsiveness was further illustrated when the immaturity of DC was preserved by removing TNFα from day 6 to day 12 and when their maturation was synchronized by addition of TNFα, LPS or CD40L. Response to MIP-3α had strongly decreased upon 48h maturation with TNFα, LPS and CD40L. Meanwhile, the response to MIP-3β was induced by all three signals, CD40L and LPS beeing more potent than TNFα. In kinetics experiments, the response to MIP-3α decreased by 50 to 70% after only 24h of CD40 activation and was completely lost at 72h. The response to MIP-3β was already maximal after 24h of CD40 activation and required relatively high concentration of chemokine (100-300 ng/ml at 48h).

Taken together, these results establish that immature $CD34^+$-derived DC respond to MIP-3α while mature DC respond to MIP-3β.

Example 2

Responses to MIP-3α and MIP-3β parallel the expression of their respective receptors CCR6 and CCR7 on $CD34^+$-derived DC

To define the mechanisms of regulation of MIP-3α and MIP-3β responsiveness, the expression of their respective receptors CCR6 (Power, *et al.*, 1997, *J. Exp. Med.* **186**:825-835; Greaves, *et al.*, 1997, *J. Exp. Med.* **186**:837-844; Baba, *et al.*, 1997, *J. Biol. Chem.* **272**:14893-14898; Liao, *et al.*, 1997, *Biochem. Biophys. Res. Commun.* **236**:212-217) and CCR7 (Yoshida, *et al.*, 199, *J. Biol. Chem.* **272**:13803-13809) mRNA was studied by semi-quantitative RT-PCR. During DC development from $CD34^+$ HPC, CCR6 mRNA was first detected at day 6, increased up to day 10 after when it decreased and became barely detectable at day 14. In contrast, CCR7 mRNA appeared at day 10 and steadily increased up to day 14. Moreover, CD40L-dependent maturation induced progressive down-regulation of CCR6 mRNA which became almost undetectable after 72h, and up-regulation of CCR7 mRNA as early as 24h. Similar results were obtained after either LPS or TNFα-induced DC maturation. The up-regulation of CCR7 mRNA following activation was confirmed by Southern blot analysis of cDNA libraries.

In line with the migration assays, and the regulation of CCR6 and CCR7 expression, MIP-3 α induced a Ca^{2+} flux exclusively in resting/immature DC and MIP-3β in mature DC only. Maximal Ca^{2+} fluxes

were observed with 30 ng/ml of MIP-3α and 30 ng/ml of MIP-3β, on immature and mature DC, respectively.

These results show that changes in responsiveness to MIP-3α and MIP-3β are linked to the regulation of CCR6 and CCR7 mRNA expression, and suggest that CCR6 and CCR7 are the major functional receptors expressed on DC for MIP-3α and MIP-3β, respectively.

Example 3
The response to MIP-3β is also induced upon maturation of monocyte-derived DC

Monocyte-derived DC, generated by culturing monocytes in presence of GM-CSF+IL-4 for 6 days, are typically immature DC ($CD1a^{+}$, $CD14^{-}$, $CD80^{low}$, $CD86^{low}$, $CD83^{-}$) (Cella, *et al.*, 1997, *Current Opin. Immunol.* **9**:10-16; Sallusto, *et al.*, 1994, *J. Exp. Med.* **179**:1109-1118). They migrated in response to MIP-1α and RANTES but neither to MIP-3α nor to MIP-3β. The lack of response of monocyte-derived DC to MIP-3α is in accordance with the absence of CCR6 expression on those cells (Power, *et al.*, 1997, *J. Exp. Med.* **186**:825-835; Greaves, *et al.*, 1997, *J. Exp. Med.* **186**:837-844) . Upon maturation induced by TNFα, LPS, or CD40L, responses to MIP-1α and RANTES were lost while response to MIP-3β was induced. Like with $CD34^{+}$-derived DC, the response to MIP-3β correlated with the up-regulation of CCR7 mRNA expression observed upon maturation induced by TNFα, LPS or CD40L. Again, up-regulation of CCR7 occurred at early time points (3 h), after TNFR or CD40 signaling. Moreover, migration and chemokine receptor expression data were in agreement with Ca^{2+} flux results.

These results extend to monocyte-derived DC the concept that upon maturation, DC loose their responsiveness to various chemokines while they become sensitive to a single chemokine, MIP-3β.

Example 4
Peripheral blood $CD11c^{+}$ DC migrate in response to MIP-3β after maturation

The chemotactic activities of MIP-3α and MIP-3β on immature $CD11c^{+}$ DC isolated from peripheral blood (or tonsils) also were studied. Freshly isolated DC did not migrate in response to MIP-3α, nor to MIP-3β, an observation which correlates with the absence of CCR6 and CCR7 mRNA

expression in these cells. However, the maturation which is known to occur after overnight culture with GM-CSF , turned on the response of CD11c+ DC to MIP-3β but not to MIP-3α. Once more, the response to MIP-3β correlated with the induction of CCR7 mRNA expression.

Therefore, even though immature CD11c+ DC freshly isolated from blood cannot respond to MIP-3α, these results show that the maturation dependent on responsiveness to MIP-3β also applies to *ex-vivo* isolated DC.

Example 5

In vivo MIP-3α is expressed in inflammed epithelium and MIP-3β within T cell rich areas of tonsils

The physiological relevance of the findings reported in Example 4 was addressed through the analysis of MIP-3α and MIP-3β mRNA expressions by *in situ* hybridization on sections of inflammed tonsils. mRNA for MIP-3α was detected at high levels in inflammed epithelial crypts but not in T cell rich areas nor in B cell follicles. In fact, MIP-3α expression was restricted to cells lining the epithelial crypts. In contrast, expression of MIP-3β mRNA was restricted to T cell rich areas. The strongest signal was present in scattered cells, with a distribution overlapping that of IDC. Outside the paracortical area, no signal could be detected in B cell follicles, nor in epithelial crypts. Serial sections showed clear absence of MIP-3β expression within epithelial crypts where MIP-3α was abundantly present. Sense probes for MIP-3α and MIP-3β, did not generate background hybridization.

Therefore, MIP-3α expression is restricted to inflammed epithelium, at the site of antigen entry where immature DC should be recruited. In contrast, MIP-3β is only detected in paracortical areas, where mature IDC home and generate primary T cell responses.

Example 6

Chemokine (MIP-3α) administrationin an in vitro mouse model

Since MIP-3α was shown by the inventors to be a chemotactic factor for mouse immature dendritic cells *in vitro*, the ability of the chemokine MIP-3α to attract immature DC *in vivo* and to modulate the antigen-specific immune response against a tumor *in vivo* was studied. If a tumor-associated antigen is delivered at the same time, more DC will be available

to capture the antigen, an therefore the antigen-specific response against this antigen should be increased.

Chemokine was delivered *in vivo* via a plasmid vector (PcDNA3, InVitrogen), that contains the cDNA encoding mouse MIP-3α under the control of the CMV promoter (PMIP-3α). The antigen used was β-galactosidase isolated from *E. coli*. The antigen was delivered in vivo via the same plasmid vector PcDNA3 (called Placz). The tumor was a C26 colon carcinoma syngeneic in BALB/c mice that has been stably transfected with the gene encoding for β-galactosidase. Therefore, in this system, β-galactosidase defines a tumor-associated antigen.

Groups of 6 female 6 week-old mice were injected with either the empty PcDNA3 plasmid (negative control), the plasmid Placz encoding the antigen alone, or a mixture of Placz and PMIP-3α. Injections (50 μg of total plasmid) were performed in the hind footpad every week for 4 weeks. After that time, mice were injected subcutaneously with the C26 tumor cell line expressing β-galactosidase. Typically, all mice develop subcutaneous tumors after 10 days. The appearance of tumors in these groups of mice were monitored. It was found that the appearance of tumors was delayed after Placz and Placz+PMIP-α injection (Figure 1). This shows that immunization with a plasmid encoding a tumor-associated antigen has a protective effect against tumor engraftment. The delay was greater with Placz+PMIP-3α than with Placz, suggesting that the chemokine MIP-3α increases the tumor associated antigen-specific immune response when delivered with the antigen.

It is believed that a good anti-tumor response is associated with a strong T cell-mediated antigen-specific cytotoxicity (CTL activity). Therefore, the CTL activity in the same groups of mice was analyzed 30 days after tumor inoculation. Spleen cells were removed and stimulated for five days with irradiated syngeneic DC plus an immunodominant CTL peptide

derived from β-galactosidase in the presence of interleukin-2. Then their ability to lyse a cell line stably transfected with the gene encoding for β-galactosidase (P13.1) was measured, in parallel with their ability to lyse the parental cell line P815 that does not express β-galactosidase (Figure 2). This was done using different ratios of effectors (splenocytes) versus targets (P13.1 or P815). The results show that mice injected with Placz+P-MIP-3α prior to tumor challenge have a greater CTL activity than mice injected only with Placz or with PCDNA3 alone, against the tumor-associated antigen β-galactosidase.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

Claims:

1. The use of a chemokine capable of directing the migration of dendritic cells in the manufacture of a medicament for the treatment of a disease state.

2. The use of claim 1 wherein the chemokine is selected from the group consisting of MCP-3, MCP-4, MIP-1α, MIP-1β, MIP-3α, RANTES, SDF-1, Teck, DCtactin-β and MDC.

3. The use of claim 1 wherein the chemokine is capable of directing the migration of dendritic cells to the site of antigen delivery.

4. The use of claim 1 wherein the chemokine is capable of directing the migration of dendritic cells to lymphoid organs.

5. The use of claim 1 wherein the disease state is a bacterial infection, a viral infection, a fungal infection, a parasitic infection or cancer.

6. The use of claim 1 wherein the disease state is an autoimmune disease, tissue rejection or an allergy.

7. The use of claim 5 wherein the disease state is cancer selected from the group consisting of melanoma, breast, pancreatic, colon, lung, glioma, hepatocellular, endometrial, gastric, intestinal, renal, prostate, thyroid, ovarian, testicular, liver, head and neck, colorectal, esophagus, stomach, eye, bladder, glioblastoma, and metastatic carcinomas.

8. The use of claim 3 wherein the dendritic cells are immature dendritic cells.

9. The use of claim 8 wherein the chemokine is selected from the group consisting of MIP-3α, MIP-1α and RANTES.

10. The use of claim 4 wherein the chemokine is MIP-3β.

11. The use of claim 3 further comprising the use of at least one disease-associated antigen.

12. The use of claim 11 wherein the antigen is a tumor-associated antigen.

13 The use of claim 11 wherein the antigen is a bacterial, viral or fungal antigen.

14. The use of claim 12 wherein the tumor-associated antigen is selected from the group consisting of Melan-A, tyrosinase, p97, β-HCG, GalNAc, MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-12, MART-1, MUC1, MUC2, MUC3, MUC4, MUC18, CEA, DDC, melanoma antigen gp75, Hker 8, high molecular weight melanoma antigen, K19, Tyr1 and Tyr2, members of the pMel 17 gene family, c-Met, PSA, PSM, α-fetoprotein, thyroperoxidase, and gp100.

15. The use of claim 14 wherein the cancer is prostate cancer and the tumor-associated antigen is PSA and/or PSM.

16. The use of claim 14 wherein the disease state is melanoma and the tumor-associated antigen is Melan-A, gp100 or tyrosinase.

17. The use of claim 1 further comprising the use of an activating agent.

18. The use of claim 15 wherein the activating agent is selected from TNFα, RP-105, an anti-CD-40 antibody and nucleic acids containing unmethylated CpG motifs.

19. The use of claim 1 further comprising the use of a combination of GM-CSF and IL-4 in conjunction with the chemokine.

20. The use of claim 1 wherein the chemokines are administered intradermally, intramuscularly, subcutaneously, topically, or in the form of a vector.

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 99/14148

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 98 14573 A (GEN HOSPITAL CORP) 9 April 1998 (1998-04-09) page 5, line 9 -page 6, line 25 claims	1-20
X	WO 97 31098 A (HUMAN GENOME SCIENCES INC ;LI HAODONG (US); ADAMS MARK (US); LIMA) 28 August 1997 (1997-08-28) page 3, line 4 - line 10 claims	1-20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 November 1999	07/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Seegert, K

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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